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Intracellular Pteroylpolyglutamate Hydrolase from Human Jejunal Mucosa

ISOLATION AND CHARACTERIZATION*

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Human jejunal intracellular pteroylpolyglutamate hydrolase was purified 30-fold from intestinal mucosa. The apparent molecular weight of the enzyme was 75,000 by Sephadex G-200 gel filtration, and the isoelectric point was at pH 8.0. The enzyme was maximally active at pH 4.5 and was unstable at increasing temperatures. Intracellular pteroylpolyglutamate hydrolase cleaved both terminal and internal γ-glutamate linkages. In contrast, brush-border pteroylpolyglutamate hydrolase catalyzed the hydrolysis of only terminal γ-glutamate linkages. The intracellular enzyme showed greatest affinity for the complete folic acid molecule with longer glutamate chains. Subcellular fractionation studies showed the intracellular enzyme was localized in lysosomes. These data show that the properties of human jejunal intracellular pteroylpolyglutamate hydrolase are distinct from those of the brush-border enzyme but are similar to the properties of intracellular pteroylpolyglutamate hydrolase described in other tissues.

Pteroylpolyglutamate hydrolases catalyze the hydrolysis of pteroylpolyglutamates to derivatives with shorter glutamate chains. These enzymes have been described in the intestinal mucosa of several different species. Only the intracellular form of pteroylpolyglutamate hydrolase has been found in the intestinal mucosa of most animals, whereas two forms of the enzyme have been identified in human and pig intestinal mucosa. The first is associated with the brush-border membrane, and the second is soluble and in the intracellular fraction (1). We recently described the purification and properties of human brush-border pteroylpolyglutamate hydrolase and showed that this enzyme is involved in the digestion of pteroylpolyglutamate, the predominant form of dietary folate (2). Relatively little is known, however, about the properties of human intestinal intracellular pteroylpolyglutamate hydrolase and its possible role in folate metabolism. Our present objectives were to isolate and characterize intracellular pteroylpolyglutamate hydrolase from human intestinal mucosa and to compare its properties with those of the human intestinal brush-border enzyme. These data show distinct properties for each hydrolase and suggest that intracellular pteroylpolyglutamate hydrolase may play a role in cellular folate metabolism that is unrelated to the digestion of dietary folates.

EXPERIMENTAL PROCEDURES[1]

RESULTS

Physical Properties—Intracellular pteroylpolyglutamate hydrolase was purified 30-fold (Table I). The apparent molecular weight was estimated by gel filtration to be 75,000. The isoelectric point was at pH 8.0. Maximal activity of the enzyme occurred at pH 4.5 (Fig. 1*A*) and at 65 °C (Fig. 1*B*). The enzyme was unstable at 37 °C in pH 4.5 assay buffer alone (Fig. 1C). However, the linearity of the product *versus* time curve for up to 45 min (Fig. 1*D*) indicated a protective effect of the substrate at 37 °C and ensured the validity of the enzyme assays. The activity of intracellular pteroylpolyglutamate hydrolase was unaffected by dialysis against 1 mM EDTA, and the addition of 100 μM zinc acetate to the reaction mixture resulted in 15% inhibition.

Affinity for Substrate—The K_m for $PteGlu_3$,[2] determined from a Lineweaver-Burk plot, was 1.2 μM. Fig. 2 shows reciprocal plots of $PteGlu_3$ hydrolysis in the presence of varied concentrations of $PteGlu_7$. This compound was a competitive inhibitor of the reaction, with a K_i of 0.09 μM obtained from a replot of the x intercepts. $PteGlu_2$ also showed similar inhibition characteristics but had lower affinity for the enzyme, with a K_i of 1.2 μM (data not shown). The effects of various $PteGlu_n$ moieties on the activity of the enzyme is shown in Table II. Complete inhibition of $PteGlu_3$ hydrolysis was observed with $PteGlu_2$ and $PteGlu_7$ at 0.1 mM. Both PteGlu and $H_4PteGlu$ at 0.1 mM caused a 15% inhibition and at 1.0 mM, a 60% inhibition. At 1 mM, pterine and γ-diglutamate showed 50 and 30% inhibition, respectively. There was a slight inhibition by 1 mM p-aminobenzoylglutamic acid and no inhibition by 1 mM glutamic acid, α-diglutamic acid, or α-triglutamic acid (Table II).

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[1] Portions of this paper (including "Experimental Procedures" and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-0836, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: $PteGlu_3$, pteroyltriglutamic acid; $PteGlu_7$, pteroylheptaglutamic acid; PteGlu, folic acid; $PteGlu_2$, pteroyldiglutamic acid; $H_4PteGlu$, tetrahydropteroylglutamic acid; $PteGlu_n$, pteroylpolyglutamic acid; $PteGlu_2[^{14}C]glu$, pteroyldiglutamyl[^{14}C]glutamic acid; $PteGlu_2[^{14}C]gluLeu_2$, pteroyldiglutamyl[^{14}C]glutamyldileucine; HPLC, high pressure liquid chromatography.

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TABLE I

Partial purification of intracellular pteroylpolyglutamate hydrolase

Procedure	Total protein	Protein	Total activity	Specific activity	Recovery	Purification factor
	mg	*mg/ml*	*milliunits*	*milliunits/mg*	%	*-fold*
10% homogenate*	4650	17.[illegible]	733.[illegible]	0.15	100	1
30,000 × *g* supernatant	1849	8.6	559	0.3	76	2
pH precipitation	940	4.[illegible]	480	0.51	68	3.4
$(NH_4)_2SO_4$ precipitation	392	14.0	210	0.54	30	3.6
Isoelectric focusing	24.[illegible]	1.46	100	4.14	14	27.6

*Homogenate was made from 20 g of tissue.

TABLE II

Effect of different moieties of $PteGlu_n$ on $PteGlu_5$ hydrolysis

Compound	% of control activity	
	0.1 mM	1 mM
PteGlu	85	36
H_4PteGlu	85	40
$PteGlu_3$	0	0
$PteGlu_7$	0	0
p-Aminobenzoylglutamic acid	92	85
Pterine	96	47
Glutamic acid	92	97
γ-Glutamylglutamic acid	100	70
α-Glutamylglutamic acid	100	100
α-Glutamylglutamylglutamic acid	100	100

Mechanism of Hydrolysis—As shown in Fig. 3, the labeled products resulting from hydrolysis of $PteGlu_5[^{14}C]Glu$ were equal amounts of $[^{14}C]$glutamic acid and γ-glutamyl$[^{14}C]$glutamic acid. Timed incubations of the enzyme with $PteGlu_7$ showed the rapid appearance of folic acid (PteGlu), with minimal accumulation of intermediate products (Fig. 4). Incubation of the enzyme with $PteGlu_5[^{14}C]GluLeu_2$ resulted in a release of radioactivity that corresponded to 5% of the hydrolysis rate when using $PteGlu_5$ as substrate.

Subcellular Location—Using fresh tissue, intracellular pteroylpolyglutamate hydrolase was localized in the fractions enriched with mitochondria and lysosomes (Fig. 5*A*). Freezing and thawing of the tissue resulted in a similar redistribution of the lysosomal marker enzyme and intracellular pteroylpolyglutamate hydrolase (Fig. 5*B*). More than 80% of both the lysosomal marker *N*-acetylglucosaminidase and pteroylpolyglutamate hydrolase appeared in the soluble fraction. Other marker enzymes showed no changes when compared to fresh tissue.

DISCUSSION

The absorption of dietary folate is attributed in part to the activity of specific pteroylpolyglutamte hydrolases located in the intestinal mucosa. To understand the mechanisms involved in absorption of dietary folate, we have focused our studies on two pteroylpolyglutamate hydrolases in human intestinal mucosa. Recently, we reported on the purification and properties of the brush-border enzyme (2). In the present study, we have examined the properties of the intracellular pteroylpolyglutamate hydrolase to understand the possible relationship of the two enzymes in folate digestion and metabolism.

As shown in Table I, a 30-fold purification of intracellular pteroylpolyglutamate hydrolase was achieved. The enzyme has an apparent molecular weight of 75,000, optimal activity at pH 4.5, a pI of 8.0, and instability at increasing temperature. The inhibition of $PteGlu_5$ hydrolysis by $PteGlu_3$ (K_i = 1.2 μM) and $PteGlu_7$ (K_i = 0.09 μM) showed competitive inhibition patterns with Lineweaver-Burk plots, indicative of greater affinity for longer chain pteroylpolyglutamates. Inhibition of $PteGlu_5$ hydrolysis by $PteGlu_n$, and to a lesser extent by other folate derivatives, and the lack of inhibition by α-glutamates or other moieties suggest that the enzyme requires both the complete folic acid moiety and γ-glutamate linkage for activity. The enzyme is capable of cleaving both terminal and internal γ-peptide bonds since incubation of intracellular pteroylpolyglutamate hydrolase with $PteGlu_5[^{14}C]Glu$ resulted in the release of both ^{14}C-labeled glutamic acid and ^{14}C-labeled diglutamic acid. The release of radioactivity when the enzyme was incubated with $PteGlu_5[^{14}C]GluLeu_2$ and the minimal accumulation of the intermediate product with $PteGlu_7$ incubation support this conclusion. Subcellular fractionation studies using differential centrifugation demonstrated that the intracellular pteroylpolyglutamate hydrolase is located in the lysosomes.

TABLE III

Comparison of intracellular pteroylpolyglutamate hydrolase and brush-border pteroylpolyglutamate hydrolase

Property	Intracellular	Brush border
Apparent M_r	75,000	700,000
pH optimum	4.5	6.5
pI	8.0	7.2
Reducing agent requirement	Yes	No
Temperature stability	No	Yes
Metal requirement	No	Yes (Zn^{2+}, Co^{2+})
K_m for $PteGlu_5$ (μM)	1.2	0.6
K_i for $PteGlu_7$ (μM)	0.09	0.6
Mechanism of hydrolysis	Cleaves both terminal and internal linkages	Exopeptidase
Final product	PteGlu	PteGlu
Localization	Lysosome	Brush border

Comparisons of the properties of human intracellular and brush-border pteroylpolyglutamate hydrolase indicate that they are distinct enzymes (Table III). The differences between these two enzymes include molecular weight, optimum pH, temperature stability, and requirement for metal ions and a reducing agent. Both enzymes showed similar K_m values for $PteGlu_7$ and greatest affinity when both the folic acid moiety and the γ-glutamate bond were present. However, intracellular pteroylpolyglutamate hydrolase had greater affinity for folates with longer glutamate chains, whereas the brush-border enzyme had no preference for the number of glutamate residues. Whereas intracellular pteroylpolyglutamate hydrolase is capable of cleaving both internal and terminal γ-glutamate linkages, the brush-border enzyme is an exopeptidase (2).

Comparisons of human intestinal intracellular pteroylpolyglutamate hydrolase with pteroylpolyglutamate hydrolases from other mammalian tissues reveal similarities and differences. Similar properties of pteroylpolyglutamate hydrolases have been described in human liver (16), bovine liver (17), rat liver (18), hog kidney (19), guinea pig intestine (20), and rat intestine (21). In each site, the enzyme had an acidic pH optimum and was demonstrated to be lysosomal in human

liver, rat liver, and guinea pig intestine. The ability to cleave internal γ-glutamate bonds was observed in studies of pteroylpolyglutamate hydrolase isolated from bovine liver and rat intestine, whereas exopeptidase activity was observed in human liver and hog kidney. Affinity toward longer glutamate chains was observed in both bovine liver and rat intestine. Furthermore, sensitivity to sulfhydryl agents and the protective effect of reducing agents were properties of pteroylpolyglutamate hydrolase from human liver, bovine liver, and hog kidney, which suggests involvement of SH groups in activity.

The role of intracellular pteroylpolyglutamate hydrolase in the human intestinal mucosa is obscure. A possible involvement of the intracellular pteroylpolyglutamate hydrolase in the absorption of dietary folate is not excluded but would require transport of all or part of the pteroylpolyglutamates into the cell prior to hydrolysis. Intracellular pteroylpolyglutamate hydrolase may function in regulating the levels of pteroylpolyglutamates within the enterocyte since others have demonstrated the capability for synthesis of these forms of the vitamin by intestinal mucosa (22). The similarities between human intestinal intracellular pteroylpolyglutamate hydrolase and the intracellular enzyme from other mammalian tissues imply that these enzymes have similar roles in cellular folate metabolism. Furthermore, pteroylpolyglutamate is not only the preferred coenzyme for many folate-dependent enzymes in single carbon transfer reactions but also has been found to be an effective inhibitor of a number of enzymes, including thymidylate synthetase and methylene-H_4PteGlu reductase (23, 24). Others observed increased glutamylation of folate in hepatoma cells in the presence of insulin or dexamethasone (25). These observations suggest that a fairly complex regulation of pteroylpolyglutamate levels exists in the cell and implies that jejunal mucosal intracellular pteroylpolyglutamate hydrolase may play a significant physiological role in cellular metabolism.

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SUPPLEMENTAL MATERIAL TO
INTRACELLULAR PTEROYLPOLYGLUTAMATE HYDROLASE FROM HUMAN JEJUNAL MUCOSA:
ISOLATION AND CHARACTERIZATION
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EXPERIMENTAL PROCEDURES

Source of Tissue. Human jejunal mucosa was obtained from patients undergoing elective jejunoileal bypass for obesity at revision of this operation. The amount of jejunum resected at each operation did not exceed that required for the surgical procedure. After the jejunal segment was washed in ice-cold saline in the operating room, the mucosa was rapidly dissected, wrapped in parafilm, and frozen with dry ice. Each sample was stored at -70° C until further use.

Chemicals. [3H]PteGlu was purchased from Amersham. PteGlu$_2$[^{14}C]Glu, PteGlu$_2$, PteGlu$_6$, and PteGlu$_3$[^{14}C]GluGlu$_2$ were synthesized by the solid phase method (3) and provided by Dr. Carlos Krumdieck, University of Alabama. All other chemicals were obtained from commercial sources.

Assays. The activity of intracellular pteroylpolyglutamate hydrolase was measured by a modification of the charcoal adsorption method (4). The standard reaction mixture consisted of 32 μM PteGlu$_2$[^{14}C]Glu, 66 μM glutamic acid, 33 mM sodium acetate buffer at pH 4.5, and enzyme in a final volume of 0.75 ml. After incubation at 37° C for 15 min, reactions were terminated by the addition of 0.25 ml of 10% trichloroacetic acid. Unreacted substrate was adsorbed with 0.5 ml of 3% charcoal in 0.1 M acetic acid. After centrifugation, the radioactivity liberated by the enzyme was quantitated by liquid scintillation counting. Under these conditions, less than 10% of the substrate was hydrolyzed during the reaction period. To differentiate the activities of the two human jejunal mucosal pteroylpolyglutamate hydrolases in the initial mucosal homogenate, the assay was carried out in the presence and absence of 0.17 mM p-hydroxymercuribenzoate. At this concentration, p-hydroxymercuribenzoate completely inhibits intracellular pteroylpolyglutamate hydrolase and has no effect on the brush border enzyme (5). The initial activity of intracellular hydrolase is expressed as the difference of the activities measured with and without the inhibitor.

After the initial centrifugation all enzyme activity was inhibited by p-hydroxymercuribenzoate and was omitted in assays of the subsequent preparations. Protein concentration was measured by the Bradford method (6). The activity of intracellular pteroylpolyglutamate hydrolase was expressed as milliUnits per mg of protein, where one milliUnit is defined as one nmole of substrate hydrolyzed per min.

PURIFICATION

Homogenization. A 10% homogenate (w/v) was prepared from thawed mucosa in 50 mM Tris-HCl buffer, pH 7.0, using a Brinkman polytron homogenizer, followed by vacuum filtration through cheesecloth. A supernatant fraction containing intracellular pteroylpolyglutamate hydrolase was obtained after centrifugation at 30,000 x g for 15 min, and 2-mercaptoethanol was added to the supernate to a final concentration of 10 mM. Since intracellular pteroylpolyglutamate hydrolase was sensitive to oxidation, preservation of its activity required a reducing agent.

pH Precipitation. The supernate was adjusted to pH 4.5 with 12 N HCl, followed by centrifugation at 30,000 x g for 15 min. The pellets were discarded, and the pH of the supernate was adjusted to pH 7.0 with 10 N NaOH.

Ammonium Sulfate Precipitation. The supernate was concentrated by ammonium sulfate precipitation. A pellet from 40% to 80% saturation was obtained and dissolved in a minimal volume of 50 mM sodium acetate buffer containing 10 mM 2-mercaptoethanol, at pH 7.0, and subsequently dialyzed against the same buffer (100 x sample volume, 3 changes, 4 hr) to remove ammonium sulfate.

Isoelectric Focusing. The concentrated sample was applied to an LKB isoelectric focusing column (LKB 8101, 110 ml) and underwent isoelectric focusing for 48 hr in a 0-40% sucrose gradient with 2% ampholyte (pH 4.5-5.0) at a constant voltage of 800 V. Fractions containing enzyme were pooled and dialyzed against 50 x volume of 50 mM sodium acetate buffer containing 10 mM 2-mercaptoethanol, 10% sucrose at pH 7.0, with 3 changes for 4 hr to remove ampholyte. The sample was stored at 4° C and subsequently used for characterization studies.

CHARACTERIZATION

Molecular Weight Determination. Molecular weight was determined by gel filtration in Sephadex G-200 (2 x 100 cm) with blue dextran (void volume), catalase (232,000), aldolase (158,000), bovine serum albumin (67,000), and cytochrome c (13,000) as markers.

Effects of pH and Temperature. The pH and temperature dependence of pteroylpolyglutamate hydrolase activity and stability were determined over a range of conditions as detailed in Fig. 1.

Effect of Metal Ions. Standard assays for pteroylpolyglutamate hydrolase were performed after dialysis of the enzyme against 1 mM EDTA. The effect of zinc was also examined by adding zinc acetate (100 μM) to the reaction mixture using nondialyzed enzyme.

Affinity for Substrate. The kinetic constants of intracellular pteroylpolyglutamate hydrolase for PteGlu$_2$[^{14}C]Glu were determined by measuring the initial reaction velocities at various substrate concentrations. The effects of glutamate chain length on enzyme activity were studied by measuring the initial velocities of PteGlu$_2$[^{14}C]Glu hydrolysis in the presence of different fixed concentrations of PteGlu$_2$ (0-5 μM) or PteGlu$_6$ (0-0.5 μM). Substrate specificity was determined by examining the effects of various moieties of PteGlu$_3$ on activity. The reaction was measured using 4 μM PteGlu$_2$[^{14}C]Glu with the addition of 0.1 mM or 1.0 mM of PteGlu, H$_4$PteGlu, Glu, γ-Glu$_2$, α-Glu$_2$, αα-Glu$_3$, N-p-aminobenzoylglutamate, or pterine.

Mechanism of Hydrolysis. The mechanism of hydrolysis of intracellular pteroylpolyglutamate hydrolase was studied by chromatographic identification of the products of PteGlu$_2$[^{14}C]Glu using an AG1-X8 ion exchange column (Bio-Rad) and of the products of PteGlu$_6$ hydrolysis using a modification of the HPLC method with detection at 280 nm as described by Shane et al. (7). To confirm the ability of intracellular pteroylpolyglutamate hydrolase to cleave internal γ-glutamate linkages, activity was measured using the standard assay, substituting PteGlu$_3$[^{14}C]GluGlu$_2$ (50 μM) as the substrate. Release of radioactive product from this compound would require the hydrolysis of internal γ-glutamate linkages.

Subcellular Localization. A 15% homogenate (w/v) was made from either fresh or previously frozen tissue in 0.25M sucrose, 1 mM phosphate buffer, pH 7.0. After filtration through cheesecloth, subcellular fractions were obtained by differential centrifugation following the method described by Hejre et al. (8). Marker enzymes included lactate dehydrogenase for cytosol (9), sucrase and brush border pteroylpolyglutamate hydrolase for brush borders (3,10), N-acetylglucosaminidase for lysosomes (11), Na$^+$-K$^+$ ATPase for basolateral membranes (12), aryl esterase for endoplasmic reticulum (13), and succinate-INT reductase and glutamate dehydrogenase for mitochondria (14,15). The distribution patterns of intracellular pteroylpolyglutamate hydrolase were compared with marker enzymes in fresh and frozen tissue.

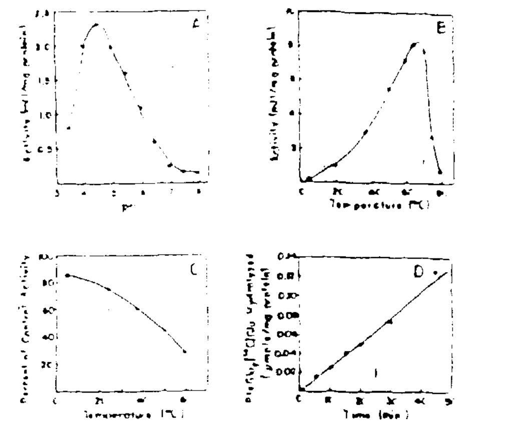


Figure 1. Dependence of intracellular pteroylpolyglutamate hydrolase on pH and temperature.

A. pH-dependent activity profile of intracellular pteroylpolyglutamate hydrolase. Enzyme was assayed in the standard reaction mixture using 33 mM 3,3-dimethylglutarate at varied pH for 15 min.

B. Temperature-dependent activity profile of intracellular pteroylpolyglutamate hydrolase. Enzyme was assayed in the standard reaction mixture at varied temperatures for 15 min.

C. Temperature stability of intracellular pteroylpolyglutamate hydrolase. Enzyme was preincubated at various temperatures in the reaction mixture without substrate for 30 min. Bovine serum albumin (0.5 mg/ml) and pepstatin (0.2 μM) were added to prevent proteolysis. After cooling on ice, enzyme activity was assayed at 37° C as described under Experimental Procedures.

D. Product production over time. Standard reactions were performed for 5, 10, 15, 20, 30, and 45 min.

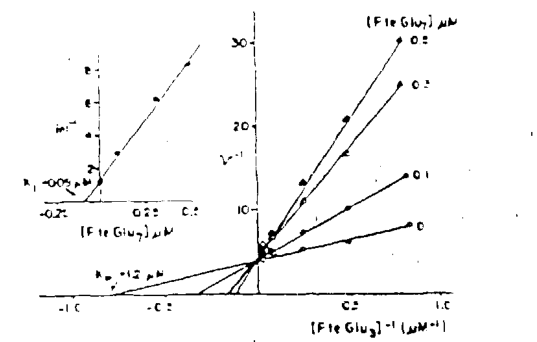


Figure 2. Inhibition of PteGlu$_3$ hydrolysis by PteGlu$_6$. The initial velocities at varied concentrations of PteGlu$_2$[^{14}C]Glu in the presence of different concentrations of PteGlu$_6$ were determined in standard reaction mixtures. The reactions were started by the addition of enzyme. Data were plotted as initial velocity^{-1} versus [PteGlu$_3$]$^{-1}$. Inset is the y-intercept of the primary plot.

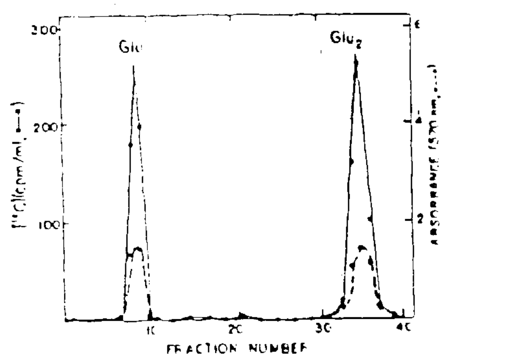


Figure 3. Glutamate reaction products of intracellular pteroylpolyglutamate hydrolase. Enzyme was incubated in the standard reaction mixture for 1 hr. The reaction was stopped with 2 ml of cold buffer containing 5 mM phosphate (pH 7.0), 0.1 mM MgCl$_2$, and 10 μmoles of glutamic acid and 5 μmoles of γ-glutamylglutamate as marker. The sample was applied to a 1 x 30 cm Bio-Rad AG1-X8 column equilibrated with 5 mM phosphate buffer (pH 7.0). The column was eluted with a 0 to 0.3 M NaCl gradient (500 ml total volume), while collecting 5 ml fractions. The markers were detected using ninhydrin reagent and radioactivity determined by scintillation counting.

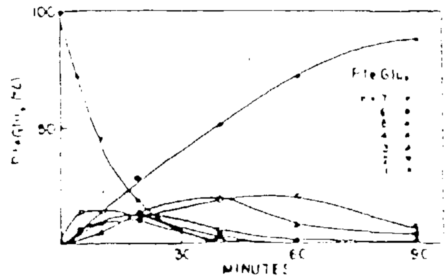
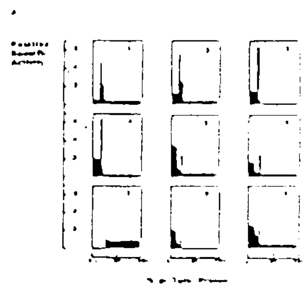


Figure 4. Pteroylglutamate reaction products of intracellular pteroylpolyglutamate hydrolase and $PteGlu_7$. Enzyme was incubated in the standard reaction mixture with 20 μM $PteGlu_7$ as substrate. The reaction was started with [illegible] milliunits of enzyme and incubated for 0, 5, 15, 30, 45, 60, and 90 min at 37° C. The reaction was terminated by placing the reaction tube in boiling water for 10 min and subsequently filtering it on a Millipore Swinnex-13 filtering apparatus. 150 μl of the filtrate was applied to a Whatman Partisil 10 SAX column for HPLC determination of products. The results are expressed as percent of total $PteGlu_7$.



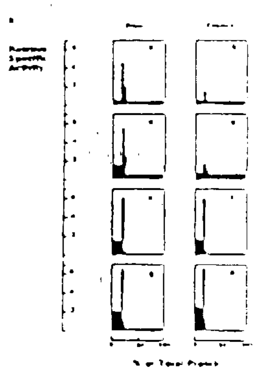


Figure 5. Distribution patterns of marker enzymes and intracellular pteroylpolyglutamate hydrolase.

A. The subcellular fractions obtained by starting with fresh tissue included A) 1,000 x g x 10 min, B) 8,000 x g x 10 min, C) 17,000 x g x 10 min, and D) 17,000 x g x 10 min supernate. Marker enzymes, as described in Experimental Procedures, were assayed for each fraction. Activity is expressed as relative specific activity (specific activity of the fraction divided by the specific activity of the original homogenate). 1. Intracellular pteroylpolyglutamate hydrolase, 2. N-acetylglucosaminidase, 3. succinate-INT-reductase, 4. glutamate dehydrogenase, 5. brush border pteroylpolyglutamate hydrolase, 6. sucrase, 7. lactate dehydrogenase, 8. aryl esterase, 9. Na^+-K^+ ATPase.

B. Comparison of distribution patterns of lysosomal and mitochondrial marker enzymes in fresh and previously frozen and thawed tissue. Figures a and b are intracellular pteroylpolyglutamate hydrolase, c and d are N-acetylglucosaminidase, e and f are succinate-INT reductase, and g and h are glutamate dehydrogenase.